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Ploszaj et al., Amino acids (Austria), 2000, 19 (2), p483-96.

Stefanelli et al. Biochemical journal (England) May 1, 2000, 347 Pt. 3, p875-80.

Sakagami et al. Anticancer Research (Greece), Jan-Feb. 2000, 20 (1A), p265-70.

Ray et al., American journal of physiology, Cell physiology (US), Mar. 2000, 278 (3), pC480-9.

Bock et al. Radiation research (US), Dec. 1999, 152 (6), p604-10.

Dai et al. Cancer research (US), Oct. 1, 1999, 59 (19), p4944-54.

6812032

Bratton et al. Jo. of biological chemistry (US), Oct. 1, 1999, 274 (40), p28113-20.

Palyi et al. Anti-cancer drugs (England), Jan 1999, 10 (10, p103-11.

Li et al. Am. journal of physiology , April 1999, 276 (4 Pt. 1), pC946-54.

Ray et al. Am. journal of physiology, Mar. 1999, 276 (3 Pt. 1) pC684-91.

8600753

Das et al. Oncology Research (US), 1997, 9 (11-12), p565-72.

Monti et al.. Life Sciences (England), 1998, 62 (9), p799-806.

Lin et al., Experimental cell research, (US), Nov. 25, 1997.

Tome et al. biochemical Journal (England) Dec. 15, 1997, 328 (Pt. 3), p847-54.

Hu et al., Biochemical journal (England), Nov. 15, 1997, 328 (Pt. 1), p307-16.

Tome et al. biological signals (Switzerland), May -Jun 1997, 6 (3), p150-6.

Taguchi et al., Cell biochemistry and function (England), Mar 2001, 19 (1), p19-26.

Camon et al. neurotoxicology (US), Fall 1994, 15 (3), p759-63.

0270-4137

Shinki et al., Gastroenterology (US), Jan 1991, 100 (1), p113-22.

D Heston et al. Prostrate (US), 1982, 3 (4), p383-9

Stefanelli et al, biochemical journal (England), Apr. 1, 2001. 355 (pt. 1), p199-206.

Lopez et al., biocell: official journal of the sociedades latinoamericanas de microscopia electronica... et. al. 9Argentina), Dec. 1999, 23 (3), p223-8.

Schipper et al. seminars in cancer biology (US), feb. 2000, 10 (1), p55-68.

Nilsson et al., biochemical journal (England) Mar. 15, 2000, 346 Pt. 3, p699-704

giuseppina monti m. et al., biochemical and biophysical research commun. (US), Apr. 13, 1999, 257 (2), p460-5.

ratasirayakorn et al, j. of periodontology feb. 1999, 70 (2), p179-84

stabellini et al., Experimental and molecular parhology (US), 1997, 64 (3), p147-55.

Sparapani et al, experimental neurology (US), nov. 1997, 148 (1), p[illegible]

Dhalluin et al., carcinogenesis (Eng.), Nov. 1997, 18 (11), p2217-23.

The Prostate 3:383–389(1982)

Copenhagen Rat Prostatic Tumor Ornithine Decarboxylase Activity (ODC) and the Effect of the ODC Inhibitor Alpha-Difluoromethylornithine

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The R3327MAT-Lu tumor is a rapidly growing anaplastic derivative of the Dunning R3327 prostatic adenocarcinoma. We have found the ornithine decarboxylase (ODC) activity of this tumor to be as sensitive to inhibition by alpha-difluoromethylornithine (DFMO) as normal rat prostate. The same was true for all the other R3327 tumor derivatives we studied. The in vivo inhibition of ODC by DFMO allowed increased uptake of exogenously administered putrescine by the R3327AT tumor. Further, DFMO was inhibitory to the growth of the R3327MAT-Lu both in vitro and in vivo.

Key words: difluoromethylornithine, suicide substrate, Dunning R3327 tumor, prostate, ornithine decarboxylase, polyamine, putrescine

INTRODUCTION

The activity of the enzyme ornithine decarboxylase (ODC) has been positively correlated with growth processes in a large number of normal or tumorous tissues [1, 2]. ODC is the rate-limiting step in the formation of the diamine putrescine, and putrescine is required for the further synthesis of the polyamines spermidine and spermine. The ability of a cell to form polyamines is assumed to be necessary for RNA and DNA synthesis. It has been shown that ODC enzymes of different tissues exhibit different properties [3–5]. Furthermore, it has been shown that the in vivo administration of the suicide substrate alpha-difluoromethylornithine (DMFO) preferentially inhibits prostatic ODC activity when compared with inhibition of ODC activities of other tissues [6–8]. Because of the preferential inhibition of the ODC activity of normal rodent prostate by DFMO, we investigated the inhibitory activity of DFMO on the Dunning Copenhagen rat prostate-derived tumor lines. We report here the results of these studies.

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MATERIALS AND METHODS

Chemicals

Alpha-difluoromethylornithine was the generous gift of the Centre de Recherche Merrell International, Strasbourg Cedex France. Alpha-methylornithine was purchased from Calbiochem, LaJolla, California. L-Ornithine and standard lab chemicals were purchased from Sigma, St. Louis, Missouri. All tissue culture media were purchased from KC Biologicals, Lenexia, Kansas. l-^{14}C-l-Ornithine,^{14}C-putrescine, and NCS tissue solubilizer were purchased from Amersham, Arlington Heights, Illinois. For protein determinations, a protein reagent kit was obtained from Bio Rad Laboratories, Richmond, California.

Animals and Tumors

All animals were inbred Copenhagen rats two to four months of age and were purchased through the Mamalian Genetics Branch of the National Cancer Institute. Thee R3327 tumor and derivative lines were maintained by techniques described previously [9].

ODC Assays

Three hours following the intraperitoneal (IP) injection of either phosphate-buffered saline (PBS, 1 ml/kg) or DFMO (100 mg/kg), tumors (1–2 cm) were surgically removed from tumor-bearing animals or normal tissues were removed from nontumor-bearing animals. A 25% tissue homogenate was prepared with the aid of a polytron tissue homogenizer in a buffer solution of 20 mM phosphate, pH 7.0, 5mM dithiothreitol, and 0.1 mM EDTA. The homogenate was centrifuged at 50,000g for two hours and the supernatant decanted and assayed for ODC activity by the CO_2-trapping procedure described by Beaven et al [10]. Protein was determined by the method of Bradford with the use of materials purchased from Bio-Rad Laboratories [11].

Putrescine Uptake Assays

Copenhagen rats bearing R3327AT tumors were divided into two groups and given either plain drinking water or drinking water containing 2% DFMO. Seventy-two hours later, the animals were injected with 1.29 μCi ^{14}C putrescine (116 mCi/mM) per 100 gm body weight IV via the penile vein. Three hours following this injection, tumor and various tissues were removed, freed of fat and connective tissue, and weighed to the nearest milligram. A 100–200 mg aliquot of tissue was taken, placed in a scintillation vial and digested overnight with 1.5 ml of NCS tissue solubilizer at 50°C. Ten milliliters of Scintillene^{00} scintillation cocktail were added to the digested samples, and the samples were counted in a Packard Tricarb liquid scintillation counter. Counts were corrected for quenching by the channels ratio technique.

Growth Inhibition In Vitro: Clonogenic Assay

The in vitro inhibitory capability of the ornithine analogs was studied by clonogenic assay as previously described [12]. R3327MAT-Lu cells were plated at initial cell density of 10^4 cells per 65-mm Petri dish. The cells were incubated for one week in MEM-media supplemented with 10% calf or rat serum with or without the addition of either 1 mM-L-ornithine, 1 mM alpha-methylornithine, or 1 mM alpha-difluoromethylornithine. Following a one-week incubation, the media was decanted, and the cell

Vivo Administered Alpha-Difluoromethylornithine (DFMO) on ODC te-Derived Tumors

ODC Activity[a]	% Inhibition with DFMO[b]	Tumor doubling time (days)[c]
4,920 ± 510[e]	91 ± 6	2
4,610 ± 470[d]	92 ± 6	2
480 ± 60	82 ± 4	5
240 ± 20	86 ± 5	20
110 ± 15	88 ± 6	—
1,400 ± 130	92 ± 5	—
47 ± 10	10 ± 4	—

s $^{14}CO_2$ released from ^{14}C-L-ornithine per hour per mg protein at room ntrols [1]. There are ten tumors or tissues per group.
e the animals were injected IP with DFMO, 100 mg/kg, and the mean percent s determined by comparison with the ODC activity of control PBS (1.0 re were ten individual tumors or tissues per each group.
s determined as previously described [9].
are the following: R3327, originally reported by Dunning slow-growing, c adenocarcinoma, R3327 HIF, androgen-independent, fast-growing adeno-3327; R3327 AT, anaplastic fast growing nonmetastatic autonomous R3327 metastatic derivative of the R3327 AT tumor that metastasizes to the lungs.

were enumerated following visualization by Papanicolaou

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mor cells were inoculated with 10^6 cells in the right and left 3]. Five animals received 1.0 ml/kg PBS IP and five animals MO IP every eight hours for 18 days following inoculation of following the final injection, the animals were sacrificed; the emoved, freed of connective tissue, and weighed to the nearest d the tissue content of DNA and RNA was determined as

ble I that the R3327 derivative line, which is an androgen-de-ell-differentiated adenocarcinoma with a doubling time of ap-an ODC activity about twice that of the normal dorsal pros-ndependent anaplastic derivative lines, the R3327AT and the DC activities that were over tenfold greater than that of the rentiated R3327 tumor, and similarly, the anaplastic tumors e ten times more rapidly.
substrate alpha-difluoromethylornithine (DFMO) when ad-/kg three hours prior to removing the tumor or tissue was oximately 90% the ODC activity of the normal prostate and ines. In contrast, the inhibition produced by this dose of

DFMO on the thymus ODC activity was only 10%. We have used the thymus here as a control tissue because a lesser degree of inhibition of the thymus ODC activity has been reported to be typical for most nonprostatic normal tissues [8].

Because we found the ODC activity of the tumor to be sensitive to the inhibitory effects of DFMO, we assayed the ability of DFMO to prevent the growth of the R3327MAT-Lu tumor in vitro with our previously described clonogenic assay [6, 12]. The results are seen in Figure 1. When 1 mM concentrations of either L-ornithine, alpha-methylornithine, or DFMO were added to the media, DFMO produced the most dramatic suppression of growth. At this concentration, L-ornithine had no effect on growth. Alpha-methylornithine, a competitive inhibitor of ODC activity, was suppressive and reduced the number of colonies by 48%. DFMO totally suppressed colony formation at this concentration, and although there were clusters of cells on the plates, all the clusters observed contained fewer than the 100 cells required to be counted as colonies.

Because we observed growth inhibition under certain conditions in vitro, we analyzed the growth inhibitory properties of DFMO in vivo. We injected 250 mg of DFMO IP every eight hours to one group of R3327MAT-Lu-bearing animals and an equal volume of phosphate-buffered saline into the other group. After 18 days of drug injections, the animals were sacrificed, and their tumors were removed, weighed, and analyzed for DNA and RNA content. These results are listed in Table II. Tumor wet weight, DNA content, and RNA content were all suppressed to nearly half of the untreated control values.

In Table III, it can be seen that when ^{14}C-putrescine is injected IV, the prostate and the R3327AT tumor retained more label than any of the other tissues such as the liver and skeletal muscle. This difference in putrescine uptake is further increased following DFMO pretreatment. DFMO enhanced putrescine uptake into the prostate-derived tumor and the prostate by nearly twofold. There was no increased putrescine uptake into either the skeletal muscle or the liver.

DISCUSSION

The Copenhagen rat prostatic tumor was originally derived from the rat dorsal prostate. Therefore, the finding that the level of ODC activity in the dorsal prostate is similar to that found in the well-differentiated, androgen-dependent, slow growing adenocarcinoma and quite different from that of the ventral prostate is in agreement with its tissue of origin [9].

In this regard, it has been reported that alterations of ODC activity in the dorsal prostate of the testosterone-stimulated castrate male rat was closely associated with the rate of DNA synthesis, while in the ventral prostate, they were dissociable, as ODC activity remained elevated long after DNA synthesis had diminished [14]. It appears that ODC activity of these prostatic tumor lines is also a reflection of cell growth as the higher the level of ODC activity, the more rapid is the tumor growth and the shorter the tumor doubling time.

The growth of the R3327 MAT-Lu tumor line was found to be inhibited in vitro the same as that found for the R3327AT from which it was derived [6]. This being the case, we wondered if DFMO would be growth inhibitory in vivo. It was, inhibitory in that this dose resulted in decreased wet weight, DNA Content, and RNA content when compared with the control group. Further, this reduction in tumor growth occurred

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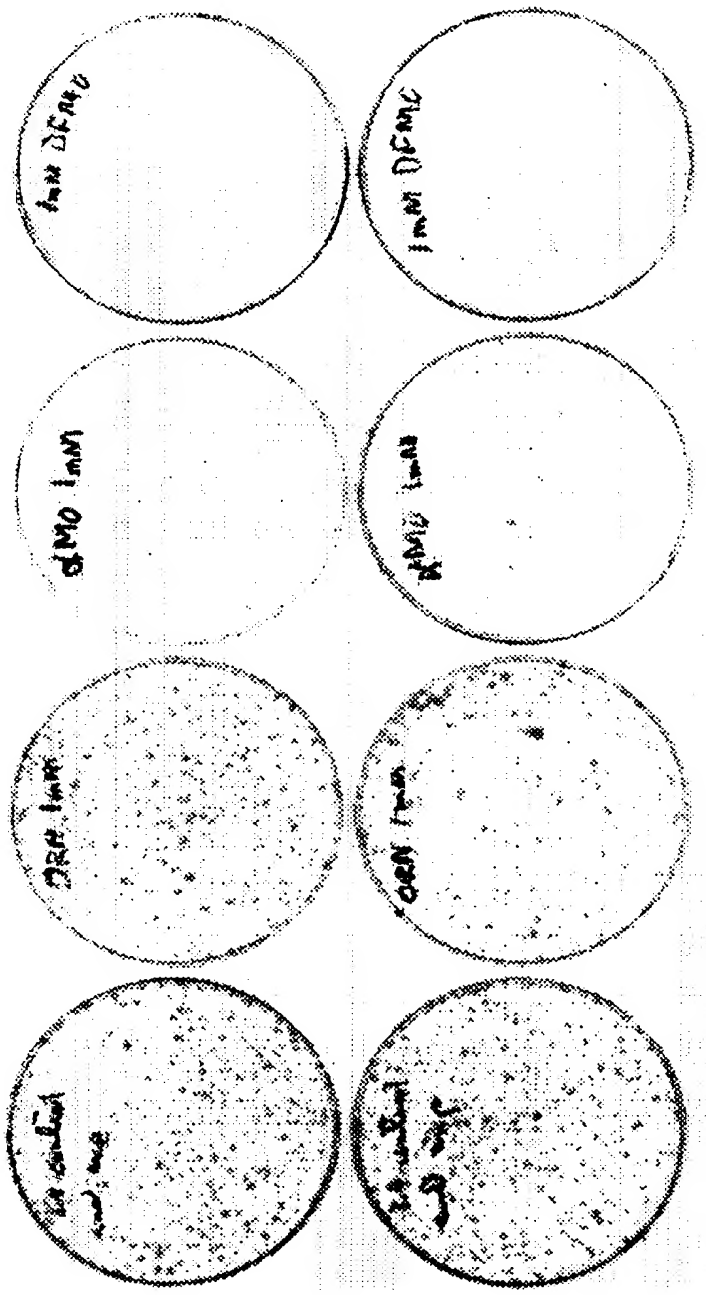

Fig. 1. In vitro clonogenic response to ornithine analog of the R3327MAT-Lu tumor. Cells were plated at 10^4 cells per dish in duplicate in the absence or presence of 1 mM ornithine, alpha-methylornithine, or alpha-difluoromethylornithine. One week later, the media was decanted, and the colonies (100 cells) were enumerated following staining with Papincolaou stain. This figure pictures, from left to right, the colonies obtained in the absence or presence of ornithine, alpha-methylornithine, and alpha-difluoromethylornithine, respectively.

TABLE II. Effect of Alpha-Difluoromethylornithine (DFMO) on the In Vivo Growth of the R3327 MAT-Lu Tumor

R3327 MAT-Lu tumor[a]	PBS-control	DFMO treatment
Tumor wet weight	1.39 ± 0.13 gm[b]	0.79 ± 0.18[c]
Total DNA content	3.72 ± 0.42 mg	1.72 ± 0.44[c]
Total RNA content	4.56 ± 0.35 mg	2.38 ± 0.46[c]

[a]One million MAT-Lu monodispersed cells were inoculated into both flanks of five control (PBS) or five treatment (DFMO) group animals. Phosphate-buffered saline (PBS) 1 ml/kg or DFMO 250 mg/kg was injected IP at eight-hour intervals for the 18-day duration of the experiment. Three hours following the last injection, tumors were surgically removed, weighed to the nearest hundredth of a gram, and assayed for DNA and RNA content.
[b]Mean of ten tumors ± SE.
[c]Statistically different from control ($P < .05$) by student's t-test.

TABLE III. Effect of Alpha-Difluoromethylornithine (DFMO) on the In Vivo Uptake of ^{14}C-Putrescine[a]

	^{14}C -Putrescine uptake (dpm/G tissue wet weight)		
Tissue[b]	Saline control	Pretreatment DFMO[c]	Enhancement ratio
R3327AT	42,450 ± 5,120	83,246 ± 9,336*	1.96
Ventral prostate	40,520 ± 8,139	65,340 ± 8,887*	1.61
Dorsal prostate	23,049 ± 2,250	45,798 ± 2,726*	1.99
Liver	14,610 ± 1,347	12,515 ± 1,857	0.86
Skeletal muscle	5,720 ± 1,220	4,720 ± 355	0.82

[a]Copenhagen rats bearing R3327AT tumors in each flank were injected IV via the penile vein with 1.29 μCi ^{14}C-putrescine (116 mCi/mM) per 100 gm body weight. Three hours following this injection, tumors and tissues were removed and analyzed for their uptake of ^{14}C-putrescine.
[b]There were ten tumors and five tissues per group.
[c]For the three days before the injection of putrescine, the animals received DFMO as a 2% solution in their drinking water.
*Statistically different from control ($P < .05$) by the Student's t-test statistic.

without any apparent toxicity to the host as the body weight of the DFMO-treated group did not decrease relative to the untreated group.

The R3327AT cells and normal prostate responded to this inhibition of ODC by increased uptake of IV injected putrescine. Others have noted that the chemotherapeutic agent methylglyoxal bis-guanylhydrazone is concentrated in tumor cells that have had their ability to synthesize polyamines blocked by DFMO treatment [15]. This increased uptake of methylglyoxal bis-quanylhydrazone by DFMO has recently been shown to be exploitable as a chemotherapeutic maneuver in humans [17]. In this hormone-insensitive tumor and in androgen-stimulated normal prostate, we find that DFMO stimulates the uptake of exogenous putrescine. This finding may be used to advantage in the design of putrescine analogs as scanning or cytotoxic agents for prostatic cancer [18].

CONCLUSION

In the treatment of prostatic cancer, DFMO may be a useful therapeutic agent to use in combination with other inhibitors of polyamine synthesis such as the

methylglyoxal bis-quanylhydrazone or with other standard chemotherapeutic agents [15, 16]. Further, it may be useful in combination with analogs of putrescine designed as either cytotoxins or for scanning.

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REFERENCES

1. Tabor CW, Tabor H: 1,4-Diaminobutane (putrescine), spermidine and spermine. Annu Rev Biochem 45:285–306, 1976.
2. Janne J, Poso H, Raina A: Polyamines in rapid growth and cancer. Biochim Biophys Acta 473:241–293, 1978.
3. Clark JL, Fuller JL: Pyroidoxal 5′-phosphate and the regulation of ornithine decarboxylase activity and stability. Eur J Biochem 67:303–314, 1976.
4. Obenrader MF, Prouty WF: Detection of multiple forms of rat liver ornithine decarboxylase activity. J Biol Chem 252:2860–2865, 1977.
5. Canellakis ZN, Theodarides TC: Stimulation of ornithine decarboxylase synthesis and its control by polyamines in regenerating rat liver and cultured rat hepatoma cells. J Biol Chem 251:4435–4441, 1976.
6. Heston WDW, Lazan DW, Fair WR: Aminoguanidine reversal of ornithine analogs on the in vitro clonogenic survival of the R3327AT prostate-derived tumor. Cancer Lett 11:323–330, 1981 (Amsterdam).
7. Danzin C, Jung MJ, Claverie N, Grove J, Sjoerdsma A, Koch-Weser J: Effects of α-difluormethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, on testosterone-induced regeneration of prostate and seminal vesicle in castrated rats. Biochem J 180:507–513, 1979.
8. Danzin C, Jung MJ, Grove J, Bey P: Effect of α-difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, on polyamine levels in rat tissues. Life Sci 24:519–524, 1979.
9. Smolev J, Heston WDW, Scott W, Coffey D: Characterization of the Dunning R3327H prostatic adenocarcinoma: An appropriate model for prostatic cancer. Cancer Treat Rep 61:273–287, 1977.
10. Beaven MA, Wilcox G, Topstra GK: A microprocedure for the measurement of CO_2 release from (^{14}C) carboxyl-labeled amino acids. Anal Biochem 84:638–641, 1978.
11. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254, 1976.
12. Rao BR, Nakeff A, Eaton C, Heston WDW: Establishment and characterization of an in vitro clonogenic cell assay for the R3327AT Copenhagen rat prostatic tumor. Cancer Res 38:4431–4439, 1978.
13. Lazan DW, Kadmon D, Heston WDW, Flair WR: Inhibition of metastasis of the R3327MAT-Lu Copenhagen rat prostatic tumor by diethylstilbestrol. Cancer Res 42:1390–1394, 1982.
14. Fuller DJM, Donaldson LH, Thomas GH: Ornithine decarboxylase activity and (^{125}I)iododeoxyuridine incorporation in rat prostate. Biochem J 150:5570–5572, 1975.
15. Alhonen-Hongisto L, Seppanen P, Janne J: Intracellular putrescine and spermidine deprivation induces increased uptake of the natural polyamines and methylglyoxal bis (guanylhydrazone). Biochem J 192:941–945, 1980.
16. Marton LJ: Polyamines and brain tumors: Relationships to patient monitoring and therapy. In Caldarera CM, Zappia V, and Bachrach U (eds): "Advances in Polyamine Therapy." 3:425–430, 1981.
17. Siimes M, Seppanen P, Alhonen-Hongisto L, Janne J: Synergistic action of two polyamine antimetabolites leads to a rapid therapeutic response in childhood leukemia. Int J Cancer 28:567–570, 1981.
18. Kadmon D, Heston WDW, Lazan DW, Fair WR: The polyamine synthesis inhibitor alpha-difluoromethylornithine (DFMO) enhances putrescine uptake into the androgen-stimulated castrated rat prostate. IRCS Med Sci 9:1153, 1981.